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SIDE-CHAIN OXIDIZED OXYSTEROLS AS METABOLIC REGULATORS IN VIVO

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Karolinska Institutet

Stockholm 2017

Published by Karolinska Institutet.

Printed by E-Print AB 2017.

ISBN 978-91-7676-595-1

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

The thesis will be defended at Barngatan 4, plan 6 (B64), Huddinge Hospital

Thursday, March 23, 2017 at 09:30 a.m.

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To my beloved family

ABSTRACT

Oxysterols are oxygenated derivatives of cholesterol characterized by a very short half-life and their ability to pass lipophilic membranes easily, and they are considered as important intermediates in the excretion pathways of cholesterol and its conversion to bile acids. Evidence has been presented that the production and flux of oxysterols in the brain may also be of some importance for cognitive functions. The two major oxysterols in the circulation of human and mouse are 24S-hydroxycholesterol (24S-OH) and 27-hydroxycholesterol (27-OH), which are formed by the cytochrome P450 enzymes cholesterol 24-hydroxylase (CYP46A1) and sterol 27-hydroxylase (CYP27A1), respectively. The two oxysterols 27-OH and 24S-OH are both strong inhibitors of cholesterol synthesis and activators of LXR *in vitro*. However, their role as physiological regulators under *in vivo* conditions is controversial.

The overall aim of this thesis was to investigate the regulatory role of side-chain oxidized oxysterols as metabolic regulators *in vivo*. In particular, we have studied the role of 24S-OH and 27-OH as regulators of cholesterol synthesis and activators of LXR. We used mouse models with increased levels of 27-OH like human *CYP27A1* overexpressor mice (CYP27A1tg) and *Cyp7b1* knockout mice (*Cyp7b1*$^{-/-}$), as well as a mouse model with no detectable levels of 27-OH in the circulation, *Cyp27a1* knockout mice (*Cyp 27a1*$^{-/-}$). The latter mice were treated with cholic acid to compensate for the reduced formation of bile acids. Using *Cyp27a1*$^{-/-}$ mice, we have also studied the possibility that 27-OH mediates the negative effects of dietary cholesterol on memory function in mice.

In **Paper I,** we studied a possible regulatory role of 27-OH and 24S-OH in the brain, using *CYP27A1*tg mice and *Cyp27a1*$^{-/-}$ mice. The levels of 27-OH were increased by approximately 12-fold in the brain of *CYP27A1*tg mice while levels of 24S-OH were decreased by about 25%, most probably due to increased metabolism by the CYP27A1 enzyme. Evidence was presented that cholesterol synthesis was increased in the brain of the two mouse models. There was no upregulation of the LXR-target genes in the brain of either of the two models. The increased synthesis in the brain of the *Cyp27a1*$^{-/-}$ mice is probably the consequence of the absence of an inhibitory effect of the flux of 27-OH into the brain. The increased cholesterol synthesis in the brain of the *CYP27A1*tg mice is probably due to the reduced levels of 24S-OH, leading to reduced inhibition of its synthesis. The results of this study are consistent with the possibility that both 24S-OH and 27-OH have a suppressive effect on cholesterol synthesis in the brain. We also conclude that 27-OH is not a general activator of LXR in this tissue.

In **Paper II**, we examined the role of 27-OH in the liver using the three mouse models described above. Only very modest effects on cholesterol synthesis and LXR target genes were observed in the three mouse models. The overall results do not support the contention either that 27-OH is an important regulator of cholesterol homeostasis, or that 27-OH is an activator of LXR-regulated genes under basal conditions in the liver.
It has been reported that treatment of mice with dietary cholesterol leads to upregulation of some LXR target genes in the liver. In a study by Chen *et al,* such upregulation of three different LXR target genes was not seen when treating mice

lacking 24S-OH, 25-OH and 27-OH with dietary cholesterol (Chen *et al.*, 2007). It was concluded that a 24-, 25- or 27-hydroxylation step mediated the effect of dietary cholesterol. We show here that similar effects were obtained when treating *Cyp27a1*$^{-/-}$ mice with dietary cholesterol, suggesting that the effects are mediated by 27-OH. Most of these effects could however not be observed at either the protein level, or at the level of enzyme activity.
The results suggest that 27-OH is a mediator of the cholesterol-induced effects on some LXR target genes when the mice are challenged with a high load of dietary cholesterol. The physiological importance of this effect is difficult to evaluate.

In **Paper III** we compared the two mouse models *CYP27A1*tg and *Cyp7b1*$^{-/-}$ mice, both of which have high levels of 27-OH in the circulation and the brain. In contrast to *CYP27A1*tg mice, the levels of 24S-OH in the brain were not decreased in the latter model, and cholesterol synthesis was not affected. This supports the proposal that 24S-OH is of regulatory importance for cholesterol synthesis in the brain. It has been reported that cholesterol synthesis is reduced in the kidney of *Cyp7b1*$^{-/-}$ mice. Cholesterol synthesis in the kidney of *CYP27A1*tg mice was not affected, however. We conclude that factors other than high levels of 27-OH are of importance for the reduced cholesterol synthesis in the kidney of the *Cyp7b1*$^{-/-}$ mice.

In **Paper IV** we tested the possibility that the negative effect of dietary cholesterol on cognition in mice is mediated by 27-OH. The negative effect of dietary cholesterol on spatial memory observed in wild-type mice was not observed in *Cyp27a1*$^{-/-}$ mice. The latter mice were also treated with cholic acid to compensate for the reduced synthesis of bile acids. Treatment with dietary cholesterol was shown to lead to reduced levels of the "memory protein" Arc (Activity-Regulated Cytoskeleton-associated protein) in the hippocampus of the wildtype mice. This effect was not seen in the hippocampus of *Cyp27a1*$^{-/-}$ mice. The results are consistent with the possibility that 27-OH is a mediator of the negative effects of dietary cholesterol on cognition.

In conclusion, our results suggest that both 24S-OH and 27-OH may be of some regulatory importance for cholesterol synthesis in the brain but not in the liver. Under normal basal conditions 27-OH does not seem to be a general activator of LXR, either in the brain or in the liver. We discuss the possibility that the different effects on cholesterol synthesis in the two organs may be related to the fact that almost all oxysterols in the brain are in the free form, whereas in the liver, most of them are esterified. In addition, we demonstrate that the negative effects of dietary cholesterol on cognition is mediated by 27-hydroxylation.

LIST OF PUBLICATIONS

I. **ALI, Z.***, HEVERIN, M.*, OLIN, M., ACIMOVIC, J., LOVGREN SANDBLOM, A., SHAFAATI, M., BAVNER, A., MEINER, V., LEITERSDORF, E. & BJORKHEM, I. 2013. On the regulatory role of side chain hydroxylated oxysterols in the brain. Lessons from CYP27A1 transgenic and Cyp27a$^{-/-}$ mice. *Journal of lipid research,* 54(4):1033-43[1].

II. HEVERIN, M.*, **ALI, Z.***, OLIN, M., TILLANDER V., JOIBARI M. M., MAKOVEICHUK E., LEITERSDORF E., WARNER M., OLIVERCRONA G., GUSTAFSSON, J. Å. & BJÖRKHEM, I. 2016. On the regulatory importance of 27-hydroxycholesterol in mouse liver. *Journal of Steroid Biochemistry and Molecular Biology*. ISSN 0960-0760, http://dx.doi.org/10.1016/j.jsbmb.2016.02.001.

III. **ALI Z.**, GUSTAFSSON, J. Å. & BJÖRKHEM, I. Two different mouse models with high levels of 27-hydroxycholesterol in kidney and brain: A comparison. *Manuscript*

IV. HEVERIN M., MAIOLI S., PHAM T., MATEOS L., CAMPORESI E., **ALI Z.**, WINBLAD B., CEDAZO-MINGUEZ A. & BJORKHEM I. 2015. 27 hydroxycholesterol mediates negative effects of dietary cholesterol on cognition in mice. *Behavioural brain research,* 278:356-9.

* Authors contributed equally to the work

[1] This article was included in **Zeina Ali's** licentiate thesis, Side-chain oxidized oxysterols as metabolic regulators in liver and brain, Karolinska Institutet, Stockholm, Sweden, 2014. ISBN 978-91-7549-455-5

OTHER PUBLICATIONS NOT INCLUDED IN THE THESIS

I. MAIOLI S., BAVNER A., **ALI Z.**, HEVERIN M., ISMAIL MA., PUERTA E., OLIN M., SAEED A., SHAFAATI M., PARINI P., CEDAZO-MINGUEZ A. & BJORKHEM I. 2013. Is it possible to improve memory function by upregulation of the cholesterol 24S-hydroxylase (CYP46A1) in the brain? *PloS one*. 8(7):e68534. doi: 10.1371/journal.pone.0068534.

II. ACIMOVIC J., LOVGREN-SANDBLOM A., OLIN M., **ALI Z.**, HEVERIN M., SCHULE R., SCHOLS L., FISCHLER B., FICKERT P., TRAUNER M. & BJORKHEM I. 2013. Sulphatation does not appear to be a protective mechanism to prevent oxysterol accumulation in humans and mice. *PloS one*. 8(7):e68031. doi: 10.1371/journal.pone.0068031.

III. ISMAIL MAM, MATEOS L, MAIOLI S., MERINO-SERRAIS P., **ALI Z**., LODEIRO M., WESTMAN E., LEITERSDORF E., GULYÁS B., OLOF-WAHLUND L., WINBLAD B., SAVITCHEVA I., BJÖRKHEM I, CEDAZO-MÍNGUEZ A. 27-Hydroxycholesterol Impairs Neuronal Glucose Uptake Through An IRAP/GLUT4 System Dysregulation. *In press.*

CONTENTS

LIST OF ABBREVIATIONS

ABCA1	ATP-binding cassette, subfamily A, member 1
ABCG1	ATP-binding cassette, subfamily G, member 1
ABCG5	ATP-binding cassette, subfamily G, member 5
ABCG8	ATP-binding cassette, subfamily G, member 8
ACAT	Acyl-CoA: cholesterol acyltransferase
AD	Alzheimer's disease
AMK	Adenosine monophosphate protein kinase
APOE	Apolipoprotein E
ARC	Activity-regulated cytoskeleton-associated protein
BBB	Blood brain barrier
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CNS	Central nervous system
CTX	Cerebrotendinous xanthomatosis
CYP27A1	Sterol 27-hydroxylase
CYP46A1	Cholesterol 24-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
DHEA	Dehydroepiandrosterone
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
FXR	Farnesoid X receptor
HDL	High-density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMGCR	3-hydroxy-3-methyl-glutaryl-CoA reductase
INSIG	Insulin-induced gene
LDL	Low-density lipoprotein
LDL-R	Low-density lipoprotein receptor
LRH1	Liver receptor homolog-1
LRP	Low density lipoprotein receptor related protein
LXR	Liver X receptor
RCT	Reverse cholesterol transport
SCAP	SREBP cleavage-activating protein
SHP	Small heterodimer partner
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
SSD	Sterol sensing domain
VLDL	Very-low density lipoprotein

1 BACKGROUND

1.1 CHOLESTEROL

Cholesterol is a hydrophobic molecule with the molecular formula $C_{27}H_{45}OH$ and four rings in its structure. It is a vital structural component of all mammalian cell membranes, and is necessary for proper membrane permeability and fluidity. In addition, cholesterol functions as an important precursor for the biosynthesis of steroid hormones and bile acids.

1.1.1 Cholesterol synthesis and its regulatory mechanisms

Cholesterol is synthesized by almost all types of mammalian cells, in particular in the liver and intestine (Dietschy *et al.*, 1993). Cholesterol synthesis occurs in five main steps illustrated in figure 1; 1) Condensation of three acetyl-CoA molecules to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) (Bloch, 1987); 2) Conversion of HMG-CoA to mevalonate. This reaction is catalyzed by the enzyme HMG-CoA reductase (HMGCR), which is the rate-limiting step in the cholesterol synthesis pathway (Goldstein and Brown, 1990); 3) Formation of the five carbon structure isopentenyl-PP from mevalonate. Isopentenyl-PP is the precursor not only for cholesterol but also for many non-steroidal isoprenoid molecules that are of importance for prenylation of different proteins within the cell; 4) Generation of the 30 carbon atom structure squalene from the isopentenyl-PP through multiple steps; 5) conversion of squalene into lanosterol, which is then converted to cholesterol through a series of reactions.

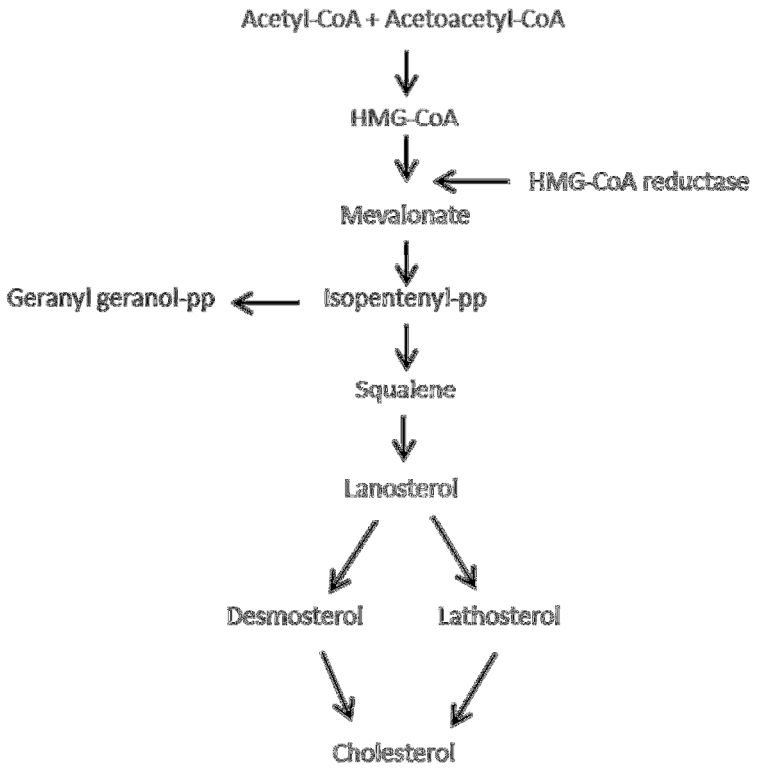

Figure 1. A simplified overview of cholesterol biosynthesis. *Cholesterol synthesis also results in production of non-steroidal isoprenoid molecules (geranyl geranol-PP). PP; pyrophosphate. The latter metabolites may be used for geranylation of a number of proteins.*

Cholesterol biosynthesis, uptake and turn-over by the cells are among the most tightly-regulated processes in cells. The rate-limiting enzyme in cholesterol biosynthesis, HMGCR, is competitively inhibited by the cholesterol-lowering drug statin (Stancu and Sima, 2001). Sterol regulatory element binding proteins (SREBPs) are endoplasmic reticulum (ER) membrane-bound transcription factors able to regulate multiple genes involved in cholesterol and fatty acids biosynthesis and uptake (Brown and Goldstein, 1999). There are three isoforms of SREBP. SREBP-1a and SREBP-1c are produced by the same gene and mainly control genes involved in fatty acid synthesis. SREBP-2 is encoded by a separate gene and controls the transcription of genes involved in cholesterol synthesis (e.g. *HMGCR*) and uptake (low-density lipoprotein-receptor (*LDL-R*)) (Brown and Goldstein, 1997). In the ER membrane, SREPB-2 is bound to the SREBP-cleavage activating protein (SCAP) which forms a complex with the anchor protein insulin-induced gene (INSIG) in the presence of high cholesterol concentrations. SCAP serves as a cholesterol level sensor due to the presence of a specific intra-membrane sequence called the sterol sensing domain (SSD). When cholesterol levels drop in the cell, the SREBP-2/SCAP complex is released from the anchor protein INSIG and transported to the Golgi, where it is subjected to enzymatic cleavage by two proteases with subsequent release of the active form of SREBP-2. The latter protein enters the nucleus and exerts its action by binding to sterol regulatory elements (SREs) in target genes (Goldstein *et al.*, 2006).

HMGCR has a sterol sensing domain (SSD) similar to that in SCAP. When the concentration of sterols increases in the ER, this will induce binding of the reductase enzyme to the ER proteins INSIG-1 and INSIG-2. This leads to recruitment of a membrane-associated ubiquitin ligase that initiates ubiquitination and subsequent degradation of the HMGCR (Sever *et al.*, 2003). The cholesterol synthesis pathway can also be rapidly regulated by phosphorylation and dephosphorylation of the enzyme HMGCR. This enzyme is most active in its unmodified form, and phosphorylation by adenosine monophosphate protein kinase (AMK) decreases its activity (Corton *et al.*, 1994; Hardie, 2003).

1.1.2 Cholesterol absorption and transportation

Cholesterol is a hydrophobic molecule, which must be associated with lipoproteins to enable its transport in the blood stream. Dietary cholesterol is absorbed from the intestinal lumen and delivered to the liver by chylomicrons. In the liver, cholesterol may be stored as cholesteryl esters by the action of the enzyme acyl coenzyme A: cholesterol acyltransferase (ACAT), converted to bile acids, secreted directly in the bile, or packed in the very low density lipoprotein (VLDL) particles. In the circulation, the triglyceride core of VLDL is subjected to lipolysis by the lipoprotein lipase and apolipoproteins A and C are transferred to high-density lipoprotein (HDL). Eventually, hydrolysis of VLDL remnants by hepatic lipase will lead to formation of low-density lipoprotein (LDL), which contains a relatively high cholesterol content. LDL delivers cholesterol to target tissues through interaction with the LDL-R.

1.1.3 Cholesterol elimination

Cholesterol is essential for the cells, but at the same time excess free cholesterol is toxic. Thus it is important to keep cholesterol levels under control by different mechanisms. Cholesterol is converted to cholesterol esters by the action of the enzymes ACAT and lecithin: cholesterol acyltransferase (LCAT) in cells and in the circulation, respectively. The unesterified cholesterol is transported to the liver in HDL particles. Lipidation of HDL is facilitated by the transmembrane ATP-binding cassette transporter A1 (ABCA1). HDL is then taken up by the liver, where the cholesterol is metabolized. The flux of excess cholesterol from the peripheral tissues to the liver by HDL is called reverse cholesterol transportation (RCT).

Another major route for elimination of excess cholesterol from the body is by secretion into the bile (via ABCG5/ABCG8) as free cholesterol for subsequent elimination in the faeces (Graf *et al.*, 2003). Extrahepatic tissues can also convert cholesterol into a hydroxycholesterol by the action of the cytochrome P-450 enzyme CYP27A1 (*see below*). This 27-hydroxylation of cholesterol allows rapid passage through the cell membrane followed by transportation in the lipoproteins back to the liver.

1.2 BILE ACIDS

Bile acids are necessary for emulsification and absorption of dietary lipids, cholesterol and lipid-soluble vitamins in the intestine. The synthesis of bile acids takes place in the liver, where approximately 500 mg of cholesterol is converted into bile acids daily. After secretion of the bile into the intestine about 95% of the bile acids are reabsorbed and returned to the liver in what is called enterohepatic circulation. About 300-500 mg of bile acids avoids this cycle and are excreted in the faeces daily. The liver compensates for this loss by replacing it with newly synthesized bile acids (~500mg/day), and this is one of the two major mechanisms by which excess cholesterol is eliminated from the body.

The enzymatic conversion of cholesterol into its oxygenated derivatives and eventually to bile acid is mainly catalysed by a group of microsomal and mitochondrial enzymes named the cytochrome P450s (Norlin and Wikvall, 2007). These enzymes are monooxygenases, possess a heme molecule as a cofactor, and use NADPH as an electron source (Danielson, 2002).

The process of bile acid synthesis involves multiple enzymes and takes two major pathways, the classical (neutral) and the alternative (acidic) (Russell, 2003). The first step in the classical pathway is catalyzed by the rate limiting enzyme cholesterol 7α-hydroxylase (CYP7A1) resulting in the production of cholic acid (CA) and chenodexoycholic acid (CDCA) with CA as the major product. In the alternative pathway CDCA is formed predominantly. The alternative pathway is initiated by the sterol 27-hydroxylase (CYP27A1) and is believed to contribute to about 25% of the formation of bile acids in rodents. Knockout of the gene *Cyp27a1* in mice results in a severe reduction in bile acid synthesis (by about 70%). As a consequence of decreased levels of bile acids, these mice also show decreased intestinal cholesterol absorption and increased cholesterol synthesis. As a compensatory mechanism, *Cyp7a1* is

upregulated in mutant mice (Rosen *et al.*, 1998; Repa *et al.*, 2000b). Feeding with cholic acid normalizes the metabolic disturbance in these mice. Unlike mice, a mutation in the human gene *CYP27A1* results in accumulation of xanthomas in the brain and tendons. This rare disease is called cerebrotendinous xanthomatosis (CTX), and is characterized by dementia, ataxia and cataracts (Bjorkhem and Leitersdorf, 2000).

The bile acid pool is auto-regulated by the binding of bile acids to the nuclear receptor farnesoid X receptor (FXR) in the liver (Makishima *et al.*, 1999; Chiang, 2004). FXR inhibits CYP7A1 transcription by a mechanism involving activation of small heterodimer (SHP) and liver receptor homologue 1 (LRH1). Bile acids fluxing into the intestine regulate the plasma levels of intestinal fibroblast growth factor (FGF) 19 (in mice, FGF15) that in turn regulates the activity of the enzyme CYP7A1 (Inagaki *et al.*, 2005; Lundasen *et al.*, 2006).

1.3 CHOLESTEROL METABOLISM IN THE BRAIN

The brain is very rich in cholesterol and its total cholesterol content is estimated to be about 25% of the total cholesterol in the body. Almost all of it exists as unesterified cholesterol. Most of the brain cholesterol (about 70%) is localized to myelin. The myelin sheath is essential for the proper functioning of the brain. Unlike other tissues in the body, the brain cholesterol pool is totally isolated from other pools in the body due to the blood-brain barrier (BBB), which is impermeable to lipoprotein-bound cholesterol in the circulation. All cholesterol found in the brain is thus synthesized *in situ*. Oligodendrocytes, the cells responsible for myelination, and astrocytes are the major cells responsible for cholesterol synthesis in the brain (Bjorkhem and Meaney, 2004).

Cholesterol synthesis in the brain is relatively high during the developmental period but reaches a very low level in the adult state. As a consequence, the cholesterol in the adult human brain has a long half-life that estimated to be about five years (Bjorkhem *et al.*, 1998; Dietschy and Turley, 2004). In spite of this long half-life, the brain still needs to eliminate some of its cholesterol to maintain constant levels. About 1-2 mg/day of cholesterol bound to apolipoprotein E (APOE) is excreted from the brain into the cerebrospinal fluid (CSF). However, the conversion of cholesterol into 24S-hydroxycholesterol (24S-OH) is considered to be the major mechanism for elimination of excess cholesterol from the brain (see below). The amount of cholesterol eliminated from the human brain through this pathway is estimated to be about 6-7mg/day (Bjorkhem *et al.*, 1998).

Neuronal cells are able to synthesize cholesterol but this synthesis is reduced during maturation. Therefore neurons are dependent on cholesterol provided by astrocytes (Pfrieger, 2003). Astrocytes are also considered to be the main producer of APOE in the brain, which is the main transporter protein for cholesterol in the central nervous system. APOE-bound cholesterol is taken up by neurons and other cells via LDL-R and the LDL receptor-related protein (LRP). Astrocytes also express the membrane transporter ABCA1, which is also important for cholesterol flux in the brain (Bjorkhem and Meaney, 2004). There are several studies in the literature linking abnormal

cholesterol metabolism in the central nervous system (CNS) to the development of neurodegenerative diseases such as Niemann-Pick C disease, Huntington's disease, Alzheimer's disease (AD) and Parkinson's disease (Block *et al.*, 2010; Madra and Sturley, 2010; Wang *et al.*, 2011), indicating the importance of normal and well-controlled cholesterol homeostasis in the CNS.

1.4 SIDE-CHAIN OXIDIZED OXYSTEROLS

Oxysterols were first identified by Lifschütz (Gill *et al.*, 2008) about one century after the characterization of cholesterol. They are oxygenated derivatives of cholesterol and can be formed enzymatically and non-enzymatically. They possess a very short half-life and the ability to pass lipophilic membranes more easily than cholesterol, making them easily redistributed in the body (Lange *et al.*, 1995; Meaney *et al.*, 2002). Oxysterols are important intermediates in cholesterol excretion pathways and conversion into bile acids. In plasma, they are associated with albumin or plasma lipoproteins. Similar to cholesterol, oxysterols are known substrates for ACAT and LCAT in cells and plasma respectively (Brown and Jessup, 2009). Oxysterols are predominantly found in the esterified form in the circulation and most organs, except for the brain, in which they are predominantly in the free form. Sulfation of oxysterols by the enzyme cholesterol sulfotransferase (SULT2B1b) is another important route for in-activation and elimination of free oxysterols (Chen *et al.*, 2007).

In addition to their important role in cholesterol transportation and elimination, oxysterols are involved in different pathological processes, such as atherosclerosis, neurodegeneration, inflammation and immunity, osteoporosis, and cancer. In this thesis, we are focusing on enzymatically-formed oxysterols, named "side-chain oxidized oxysterols", such as 24S-, 25-, and 27-OH, which are hydroxylated on their side chain. The two major oxysterols in the circulation of man and rodents are 24S-OH and 27-OH (Bjorkhem, 2009).

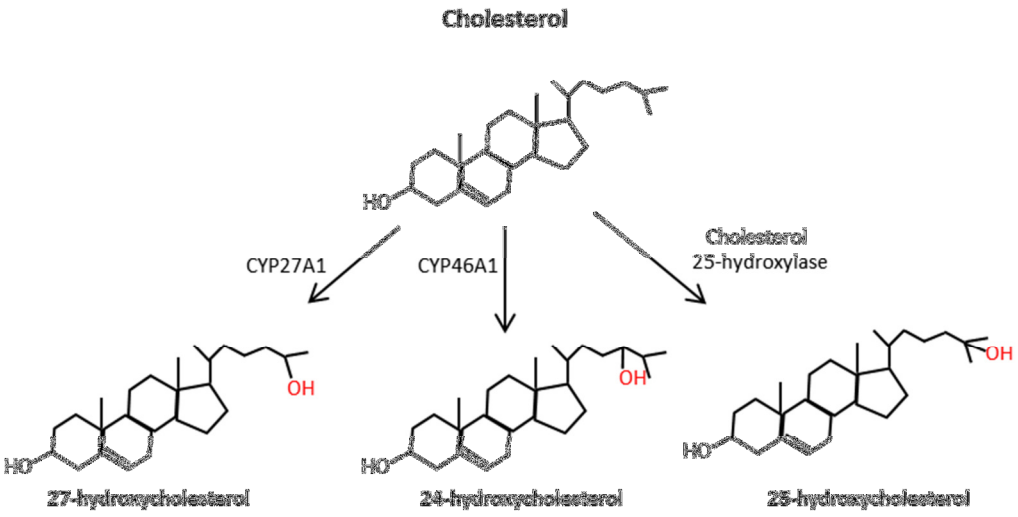

Figure 2. Structure and generation of the major side-chain oxidized oxysterols from cholesterol.

1.4.1 24S-hydroxycholesterol

Conversion of brain cholesterol to 24S-OH by the microsomal enzyme cholesterol 24-hydroxylase (CYP46A1) is considered to be the main mechanism for the elimination of excess cholesterol from the brain in both man and rodents (Bjorkhem *et al.*, 1998; Lund *et al.*, 1999). The enzyme CYP46A1 has been crystallized and the structure has been elucidated in detail (Mast *et al.*, 2008). A number of strong inhibitors and modest activators of the enzyme has been identified. CYP46A1 was found to have a broad substrate specificity including not only steroids but also a number of drugs. This enzyme is mainly expressed in the neuronal cells of the brain (Lund *et al.*, 1999) with little or no specific transcriptional regulatory mechanism (Ohyama *et al.*, 2006). Unlike cholesterol, this monooxygenated molecule, 24S-OH, is able to cross the BBB to the circulation, where it is taken up by the liver and converted into bile acids and conjugates of un-metabolized or partially metabolised 24S-OH (Bjorkhem *et al.*, 2001).

Neurons are believed to depend on astrocytes for their cholesterol supply, which is delivered in an APOE-bound form. Based on *in vitro* studies, it has been suggested by Pfrieger that the flux of cholesterol from astrocytes to neurons is regulated by 24S-OH, which is considered as one of the most efficient activators of LXR *in vitro* (*see below*). Flux of 24S-OH from neurons to astrocytes would be expected to result in transcription of the LXR-target genes *ABCA1*and *APOE* in glial cells, with a subsequent increase in cholesterol efflux (Pfrieger, 2003). This contention is supported by another *in vitro* experiment demonstrating that 24S-OH induces expression of *APOE* and APOE-mediated efflux of cholesterol via an LXR pathway (Abildayeva *et al.*, 2006).

1.4.2 27-Hydroxycholesterol

27-OH is formed by the action of the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1), which is widely expressed throughout the body. Under in vitro conditions, *CYP27A1* is regulated by several molecules, such as cyclosporine A, cholic acid (Segev *et al.*, 2001), thyroid hormone, glucocorticoids and growth hormone (Araya *et al.*, 2003). Moreover, the expression of *CYP27A1* is affected by estrogen and androgens (Tang *et al.*, 2007). However, the main regulatory factor for *CYP27A1* expression *in vitro* as well as *in vivo* is the substrate availability (Pandak *et al.*, 2002). There is a constant flux of 27-OH and metabolites of this oxysterol from extrahepatic tissues to the liver, where it is metabolized into bile acids (Lund *et al.*, 1996). At the same time, there is significant uptake of 27-OH by the brain, (approximately 5 mg/day), in which it is rapidly metabolized into the steroid acid, 7α-hydroxy-3-oxo-4-cholestenoic acid (Heverin *et al.*, 2005). This acid is also able to cross the blood-brain barrier, and is rapidly eliminated from the brain and metabolized in the liver (Meaney *et al.*, 2007). The conversion of cholesterol to 27-OH is considered to be a significant form of reverse cholesterol transport, and the absence of this mechanism in CTX patients due to absence of the enzyme CYP27A1 contributes to the premature atherosclerosis seen in these patients (Bjorkhem *et al.*, 1994).

Moreover, 27-OH is known to be the most prevalent oxysterol in the circulation and its levels are closely correlated with those of cholesterol in the circulation (Harik-Khan

and Holmes, 1990; Babiker *et al.*, 2005) . Therefore, it has been suggested that a number of negative effects of cholesterol in periphery are mediated by its metabolite 27-OH. It has been reported that 27-OH is an endogenous selective estrogen receptor modulator (Umetani *et al.*, 2007; DuSell *et al.*, 2008) which exhibits an antiestrogenic action on the vascular endothelium. Furthermore, studies in mouse models of breast cancer revealed that 27-OH enhances both tumour growth in an estrogen receptor-dependent manner, and also the metastatic effect of the cancer cells in an LXR-dependant manner (Nelson *et al.*, 2013). DuSell *et al.* demonstrated that increasing concentrations of 27-OH have a negative effect on bone homeostasis, since it causes decreased bone formation and increased bone resorption (DuSell *et al.*, 2010).

1.5 EFFECTS OF THE SIDE-CHAIN OXIDIZED OXYSTEROLS ON COGNITION AND MEMORY

Unlike cholesterol, side-chain oxidized oxysterols are able to cross the BBB. As mentioned above, 24S-OH is the major cerebral oxysterol and its plasma levels can be a used as a marker for the CNS neuronal mass. Patients with advanced AD have reduced levels of plasma 24S-OH (Bretillon *et al.*, 2000; Kolsch *et al.*, 2004). The levels of 24S-OH are also reduced in chronic multiple sclerosis patients (Leoni *et al.*, 2002; Teunissen *et al.*, 2003). Continuous production of 24S-OH is important for memory function as indicated by impaired spatial and motor learning, as well as impaired hippocampal long-term potentiation (LTP), in mice lacking the gene *Cyp46a1* (Kotti *et al.*, 2006). Treatment of wild-type hippocampal slices with statins also impairs LTP, while treatment with the non-steroid isoprenoid geranylgeraniol, a by-product of the mevalonate pathway, normalizes LTP. From these observations, the authors suggested that CYP46A1-mediated cholesterol turnover in the brain is necessary to ensure adequate production of geranylgeraniol for normal brain function. In line with this, studies in old female mice with overexpression of human *CYP46A1* showed better spatial memory, compared to controls (Maioli *et al.*, 2013). The levels of lanosterol are increased in the brains of *CYP46A1* overexpressor mice, indicating an increased cholesterol synthesis with higher production of geranylgeraniol. Another benefit of 24S-OH in the brain is its inhibitory effect on the formation of Aβ peptide. Thus 24S-OH favours the non-amyloidogenic pathway in neuroblastoma cells, while 27-OH antagonizes this effect and promotes production of Aβ (Prasanthi *et al.*, 2009). Heverin *et al.*, reported that the brain of aged mice expressing the Swedish Alzheimer mutation, and the brain of AD patients, contain increased 27-OH and decreased 24-OH (Heverin *et al.*, 2004). In light of this, overexpression of *CYP46A1* in the hippocampus of an AD model, APP23 mice, markedly reduces Aβ peptides, amyloid deposit, and it also improves spatial memory (Hudry *et al.*, 2010). Using a similar approach, overexpression of *CYP46A1* again demonstrates positive effects in both another AD model that expresses Tau pathology, and also in a model of Huntington's disease (Burlot *et al.*, 2015; Boussicault *et al.*, 2016). Furthermore, 24S-OH has been shown to be a potent allosteric modulator of N-methyl-D-aspartate receptors (NMDAR) that play a critical role in regulating synaptic plasticity (Paul *et al.*, 2013).

Mid-life hypercholesterolemia is a risk factor for AD (Kivipelto *et al.*, 2001; Solomon *et al.*, 2009), in spite of the fact that the cholesterol pool in the brain is totally isolated from the cholesterol pool in the circulation. Thus, it has been suggested that the negative effect of hypercholesterolemia on the CNS is mediated by the cholesterol oxygenated derivative 27-OH, which is able to cross the BBB. (Bjorkhem *et al.*, 2009). In line with this, Mateos *et al.* reported that dietary cholesterol alters the expression of many genes in the brain. Among those genes with altered expression, these scientists investigated the gene encoding for the ”memory protein” activity-regulated cytoskeleton-associated protein (ARC), which is found to be significantly reduced in the cortex and hippocampus after cholesterol feeding (Mateos *et al.*, 2009). This protein is normally expressed in the principal neurons in the cortex and hippocampus (Vazdarjanova *et al.*, 2006) . ARC expression increases during learning and memory training activities, thus it is an important protein in synaptic plasticity and memory consolidation (Guzowski *et al.*, 2000; Bramham *et al.*, 2010). In contrast, the expression of this ARC protein is reduced in the brain of AD patients (Ginsberg *et al.*, 2000; Mateos *et al.*, 2009). Treatment of hippocampal primary neuronal cells with 27-OH inhibits *Arc* expression (Mateos *et al.*, 2009). In addition, very recently Ismail *et al.* reported that CYP27A1 overexpressor mice, characterized by elevated plasma and tissue levels of 27-OH, exhibit decreased glucose metabolism in the brain as well as memory deficits. These mice also have decreased expression of *Arc* in both cortex and hippocampus (Ismail M.A.M., 2017). Cumulatively, these results support the contention that part of the negative effect of dietary cholesterol on memory and *ARC* expression could be mediated by 27-OH.

In light of the beneficial effects of 24S-OH and the opposite effects of 27-OH, the balance between these two steroids is likely to be of importance for neurodegenerative processes in the brain.

1.6 REGULATORY ROLES OF THE SIDE-CHAIN OXIDIZED OXYSTEROLS IN CHOLESTEROL HOMEOSTASIS

More than 30 years ago, based on *in vitro* studies, it was hypothesized that oxysterols are important physiological regulators of cholesterol homeostasis (Kandutsch *et al.*, 1978). Since then, this oxysterol hypothesis has been developed as knowledge of cholesterol homeostasis has advanced. Based on *in vitro* experiments, it’s been suggested that side-chain oxidized oxysterols reduce cellular cholesterol levels by their action at multiple points in the cholesterol homeostasis system, such as reducing cholesterol synthesis and uptake, and stimulating cholesterol efflux from the cell. Figure 3 demonstrates the different mechanisms by which oxysterols are able to limit intracellular cholesterol levels.

1.6.1 Enhanced degradation of HMGCR protein:

Oxysterols can regulate the enzyme HMGCR at the post-transcriptional level. Accumulation of oxysterols in the endoplasmic reticulum triggers the formation of the HMGCR/INSIG complex that leads to ubiquitination and subsequent degradation of the

enzyme, resulting in a rapid inhibition of cholesterol synthesis(Sever *et al.*, 2003; Goldstein *et al.*, 2006; Jo and Debose-Boyd, 2010). It has been reported that 27-OH enhances HMGCR ubiquitination and degradation under *in vitro* conditions (Radhakrishnan *et al.*, 2007). Also Lange *et al.* showed that endogenous 27-OH is important for acute proteolytic inactivation of HMGCR in response to high levels of cholesterol (Lange *et al.*, 2008). The half-life of the enzyme HMGCR dramatically reduces from longer than 12 hours in sterol-deprived cells, to less than 1 hour in cells with high sterol levels (Gil *et al.*, 1985; Goldstein *et al.*, 2006).

1.6.2 Inhibition of the SREBP pathway:

Cholesterol synthesis is regulated by the controlled movement of SREBP from the ER to the Golgi, where it is subjected to a series of cleavages, with subsequent release of the active form and its translocation into the nucleus. This movement is inhibited by either cholesterol itself or by oxysterols, thus stopping cholesterol synthesis. Cholesterol induces conformational changes in SCAP, and as a consequence SCAP binds to INSIG. On the other hand, oxysterols inhibit cholesterol synthesis by binding to INSIG, followed by binding of INSIG to SCAP. The latter binding prevents transportation of the SREBP/SCAP complex to the Golgi and subsequent lipogenic gene expression (Radhakrishnan *et al.*, 2007).

1.6.3 Activation of LXR signaling:

Some of oxysterol's effects on cholesterol homeostasis are believed to be mediated by the nuclear liver X receptor (LXR). The two LKR isoforms LXRα and LXRβ form heterodimers with retinoid X receptors (Willy *et al.*, 1995). LXRs have an important physiological role in cholesterol and lipid homeostasis, as shown by several studies in mice lacking LXR (Peet *et al.*, 1998; Schuster *et al.*, 2002). In rodents, pharmacological activation of LXR results in an upregulation of fatty acid synthesis genes e.g. *Srebp-1c, Fas*, the cholesterol efflux genes *Abca1, Abcg5, Abcg8*, the bile acid synthesis gene *Cyp7a1*, and also a downregulation of the *Cyp7b1* gene that encodes for the main enzyme in the alternative bile acid synthesis pathway (Uppal *et al.*, 2007). Activation of the LXR can be regarded as a defence mechanism preventing accumulation of excess cholesterol in tissues.

Lehmann *et al.* demonstrated that oxysterols are natural ligands for LXR under *in vitro* conditions (Lehmann *et al.*, 1997). It has been found that 24(S), 25-epoxycholesterol, 24S-OH, and 25-OH are the most potent LXR-activating oxysterols. 27-OH is a less potent activator of LXR than the aforementioned oxysterols, but this oxysterol is present at relatively high levels and has therefore been suggested as a physiological activator of LXR. Several *in vitro* studies have demonstrated the ability of 27-OH to activate the LXR-responsive genes, *ABCA1, ABCG1*and *APOE* (Kim *et al.*, 2009). Fu and colleagues also reported that 27-OH is an activator of *ABCA1* and *ABCG1* genes in cholesterol-loaded fibroblasts (Fu *et al.*, 2001).

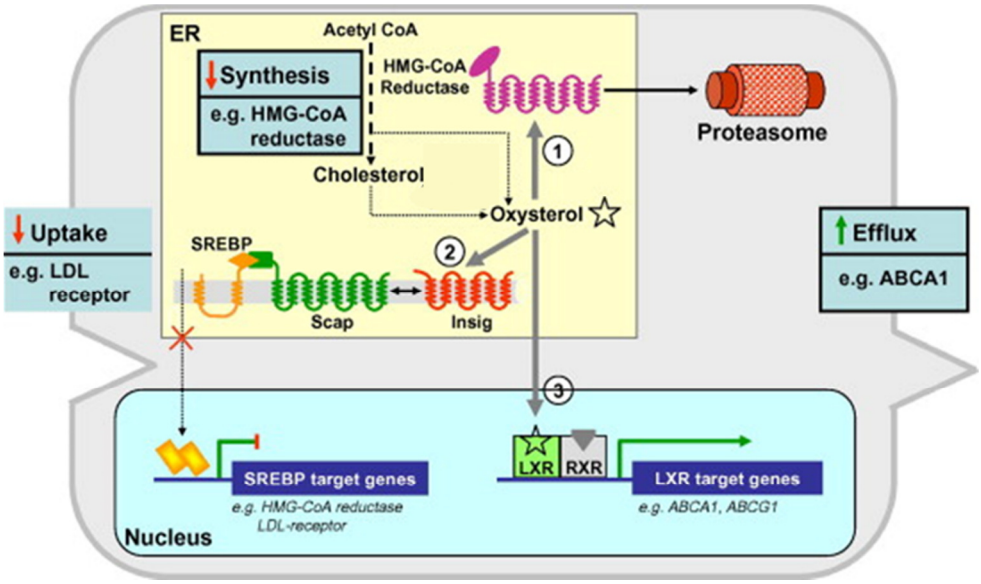

Figure 3. Regulation of cholesterol homeostasis by oxysterols. *The different mechanisms of how side-chain oxidized oxysterols reduce cholesterol status in the cell by several molecular mechanisms: 1. accelerated degradation of HMG-CoA reductase; 2. suppression of SREBP activation; 3. increased cholesterol efflux by activating LXR-mediated gene transcription. Reproduced from Brown, A. J. Jessup, W. Oxysterols: Sources, cellular storage and metabolism, and new insights into their roles in cholesterol homeostasis. Molecular aspects of medicine (Brown, 2009).*

1.7 *IN VIVO* STUDIES IN MICE WITH DIFFERENT LEVELS OF SIDE-CHAIN OXIDIZED OXYSTEROLS

The important role of oxysterols in bile acid synthesis and in cholesterol transportation is well documented. However, the physiological role of oxysterols as regulators of cholesterol homeostasis *in vivo* is still unclear. It should be emphasized that the above mechanisms for regulation of cholesterol homeostasis have been demonstrated *in vitro*, and the importance of oxysterols in the regulation of cholesterol homeostasis *in vivo* in the presence of excess cholesterol, is not known with certainty. Oxysterols are present in trace amounts only in the circulation and tissues, and are always accompanied by a 10^3- to 10^6- fold excess of cholesterol. In most tissues, this excess of cholesterol in relation to oxysterols, might affect the ability of oxysterols to bind to LXR (Bjorkhem, 2009). In the brain, however, the ratio between a side-chain oxidized oxysterol and cholesterol is higher than 1 to 1000. Therefore, there is a higher potential for LXR-mediated regulation in the brain than in other tissues.

Experiments on mice lacking the gene encoding for the enzyme CYP46A1 revealed a reduction in cholesterol biosynthesis in the brain of mutant mice by about 40%, but not

in other organs (Lund *et al.*, 2003). This reduction in cholesterol synthesis is mainly due to reduced elimination of cholesterol from the brain via its conversion into 24S-OH. In spite of this, the total cholesterol levels in the brain of the mutant mice are normal. The effect of the knockout of the enzyme CYP46A1 on LXR-target genes in the brain was not studied. Behavioral studies in these mice demonstrate severe memory defects (Kotti *et al.*, 2006), *(see above)*.

A transgenic mouse model with high levels of 24S-OH has been previously generated by our group (Shafaati *et al.*, 2011). These mice overexpress the human enzyme CYP46A1 that converts cholesterol to 24S-OH. This overexpression induces cholesterol synthesis in these mice, with unchanged cholesterol levels in the brain. It is known from *in vitro* studies that 24S-OH is one of the most efficient LXR activators (Janowski *et al.*, 1999). Contrary to expectations, there were no stimulatory effects on LXR-target genes in the brain and liver of these transgenic mice (Shafaati *et al.*, 2011). These results are consistent with other results obtained by local overexpression of CYP46A1 in the cortex and hippocampus of APP23 mice. This group also failed to detect any activation of LXR-target genes in the brain of these mice, in spite of elevated 24S-OH (Hudry *et al.*, 2010).

Knockout of the enzyme responsible for the formation of 27-OH in mice, CYP27A1, results in an approximate 75% decrease in the bile acid pool, reduced intestinal cholesterol absorption and increased hepatic cholesterol synthesis. Cholic acid supplementation in the diet of these mice reverses all of these symptoms (Repa *et al.*, 2000b). In a separate study by our group, we found that these mice have a modest upregulation of cholesterol synthesis in the brain, as predicted by high lathosterol levels (Bavner *et al.*, 2010). This increase in cholesterol synthesis is also observed in the brains of mice treated with cholic acid, as well as in non-treated mice. This result is consistent with a possible regulatory role of 27-OH in cholesterol synthesis in the brain. However, the effects of the knockout of the gene on LXR-target genes were not studied in this model.

Overexpression of the human CYP27A1 in mice results in 3-5 fold increase in 27-OH in the circulation and extra-cerebral tissues. There is no marked effect on cholesterol homeostasis in these mice (Meir *et al.*, 2002). The most striking effect is the reduced levels of 24S-OH in the circulation of the overexpressor mice, possibly due to metabolism by the overexpressed enzyme (Bjorkhem *et al.*, 2001). Disruption of the gene oxysterol 27-hydroxylase (*Cyp7b1*) in mice also results in markedly high levels of 27-OH in the circulation and tissues (Li-Hawkins *et al.*, 2000). These mice have normal cholesterol levels in the circulation and normal cholesterol synthesis in the liver. The possible consequences of the high levels of 27-OH on the expression of LXR-target genes were never studied in either of these two models.

A triplet knockout mouse model that lacks 24S-OH, 25-OH and 27-OH in the circulation and tissues was generated previously (Chen *et al.*, 2007). Due to the consequences of the knockout of *Cyp27a1*, the mice were treated with cholic acid in the diet. Challenging these mice with a high cholesterol diet failed to induce expression of some established LXR-target genes in the liver. This result led to the conclusion that

some of the effects of cholesterol feeding on LXR-target genes are likely to be mediated by side- chain oxidized oxysterols.

The above *in vivo* studies do not support the contention that side-chain oxidized oxysterols are important for cholesterol synthesis and turnover under normal conditions, with the possible exception of the situation in the brain. Except for the situation with high levels of 24S-OH, the effects of high levels of side-chain oxidized oxysterols on LXR-targeted genes have not been studied previously.

2 AIMS

The intention of the present thesis was to use transgenic mouse models with markedly changed levels of side-chain oxidized oxysterols to evaluate if such oxysterols are of importance in the regulation of cholesterol homeostasis and LXR-target genes in different organs. The specific aims were the following:

- Exploring the role of side-chain oxidized oxysterols in cholesterol homeostasis in the mouse brain (**Paper I**).

- Investigating the effect of 27-OH on the expression of cholesterol-synthesis genes and LXR-target genes in the liver, under basal conditions and after cholesterol feeding (**Paper II**).

- Comparing the effect of 27-OH on cholesterol synthesis in the brain and kidney in two different models with elevated 27-OH (**Paper III**).

- Testing the possibility that the negative effects of cholesterol on cognition is mediated by 27-OH (**Paper IV**).

3 MATERIALS AND METHODS

The following is a brief account of materials and methods used in this thesis. For more details, please refer to the respective papers.

3.1 MATERIALS

3.1.1 Animals

All mice were 2-5 months old when experiments were conducted. The mice were specifically generated for these experiments from our on-going breeding colonies in Huddinge University Hospital Animal Facility. The colonies were maintained by breeding heterozygous pairs. The animals were housed, in Makrolon type II cages with wire tops (Tecniplast, Sweden), under standard environmental conditions with free access to food and water. All mice were euthanized by CO_2 inhalation at the same time of the morning, except for Cyp27a1 knockout mice, which were euthanized by cervical dislocation. Blood and tissues were collected and stored at -80°C.

All experiments in these studies were performed in accordance with the guide lines from the Swedish national board for Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC), and approved by the Southern Stockholm Ethical Committee.

3.1.1.1 CYP27A1 transgenic mice (CYP27A1 tg)

These mice were generated previously using the b-actin promoter (Meir *et al.*, 2002). The founders were originally obtained from our collaborator Eran Leitersdorf, and they were on C57BL/6 background. In this experiment C57BL/6 mice were used as a control group, and were purchased from Charles River Laboratories, Germany.

3.1.1.2 Cyp27a1 knockout mice (Cyp27a1$^{-/-}$)

This strain was also generated previously on a C57BL/6 background (Rosen *et al.*, 1998). Homozygous knockout mice and their corresponding wild-type controls were generated from the breeding of heterozygous mice. These mice have a deficient production of bile acids that leads to a malabsorption of cholesterol and other lipids. As a consequence of this, they have a compensatory increase in cholesterol synthesis and an increased activity of CYP7A1, corresponding to the rate-limiting step in the formation of bile acids. Treatment of the mice with cholic acid normalizes cholesterol absorption and cholesterol synthesis. Because of this, we treated the Cyp27a1$^{-/-}$ mice with either 0.05% cholic acid or 0.025% cholic acid for three months before euthanasia at the age of four months (Repa *et al.*, 2000b). In addition to cholic acid, the diet was supplemented by either 1% cholesterol for the last seven days, or with 0,05% cholesterol for the last three months before euthanasia. The different diets were obtained from Lantmännen and Harlan Teklad 2918.

3.1.1.3 Cyp7b1 knockout mice (Cyp7b1$^{-/-}$)

The *Cyp7b1*$^{-/-}$ mice used in this project were generated in another laboratory, as has been described previously (Rose *et al.*, 2001). They were on C57BL/6 background. As

for *Cyp27a1*$^{-/-}$ mice, homozygous knockout mice and their corresponding wild-type controls were generated from the breeding of heterozygous mice.

3.2 METHODS

3.2.1 Lipid extraction and analysis

Livers and brains were extracted according to Folch method. Folch solution (chloroform/methanol 2:1, v:v), 3 and 10 ml, was added to about 100 mg liver tissue, and to half of a brain or a hippocampus or cortex from half of a brain, respectively. After 24 hours at room temperature, the extracts were transferred to new vials and evaporated under argon. After evaporation, the extracts were re-dissolved in 1 ml (liver) or 10 ml (brain) Folch and stored at -20°C until required. Sterols and oxysterols were measured in the Folch extract by gas chromatography–mass spectrometry after alkaline hydrolysis using deuterium-labelled internal standards as previously described (Dzeletovic *et al.*, 1995; Acimovic *et al.*, 2009). In some experiments, the hydrolysis step was excluded in order to measure the fraction of unesterified oxysterol.

3.2.2 RNA preparation and real-time PCR

Total RNA was extracted from liver and brain tissues either by TRIzol reagent, or with RNAeasy lipid tissue Mini kits (Qiagen, CA, USA) following the manufacturer's protocol. Total RNA was reverse-transcribed using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). The relative expression levels of target genes were determined by real-time RT-PCR amplification assays (Applied Biosystems). All values were normalized either to HPRT or GAPDH mRNA concentrations. The relative quantification of gene expression was carried out using the comparative cycle threshold method, $2-\Delta\Delta Ct$ (Livak and Schmittgen, 2001). Each sample was measured in triplicate.

3.2.3 Western blotting

All Western blots were made with whole homogenates of livers. Antibodies toward ABCA1 (ab1811180), lipoprotein lipase (LPLA4) (ab21356) and Actin (ab8227) were obtained from Abcam. Antibodies toward ABCG8 protein were from Santa Cruz Biotechnology (H-300: sc-30111) and Novus Biologicals Cambridge UK (NB400-117SS), and antibodies toward SREBP1C protein were from BD Biosciences (Cat# 557036). Antibodies toward ABCG5 protein were a generous gift from Dr. Ligin You, Wake Forest University School of Medicine, Winston-Salem, NC. The incubation with primary antibodies was followed by incubation with anti-rabbit or anti-mouse immunoglobulin G (IgG) at 1:10000 dilutions and Li-COR IR Dye secondary antibody at 1:15 000 dilutions. The relative density of the immune-reactive bands was calculated from the optical density (OD) multiplied by the area of the selected band using Odyssey Fc Imager software.

3.2.4 Lipoprotein lipase activity

Frozen tissue samples were homogenized in 9 volumes of a buffer containing 0.025 M ammonia, 1% Triton X-100, 0.1% SDS, 5 IU heparin/ml and protease inhibitor cocktail

tablets, pH 8.2 (Complete Mini, Roche Diagnostics, Germany, 1 tablet/50 ml). After homogenization (Bullet Blender Blue CE, Next Advance co, USA), samples were taken for analyses of LPL activity using a commercial emulsion of soy bean triacylglycerols in egg yolk phospholipid (Intralipid 20% from Fresenius Kabi, Uppsala, Sweden), into which ^{3}H-oleic acid labeled trioleolylglycerol was incorporated by intermittent sonication for 5 min (in an ice bath). Triplicates were analyzed from each sample in a total volume of 200 µl. The incubations were carried out for 2 h at 25-°C, and the reactions were stopped by addition of organic solvents for extraction of the fatty acids by the method of Dole, as described (G.Bengtsson-Olivecrona, 1992). The enzyme activity is expressed as mU per g tissue wet weight where 1 mU corresponds to the release of 1 nmol fatty acids per min.

3.2.5 Morris water maze test

The Morris water–maze (MWM) is a circular pool constructed of grey PVC, 160 cm in diameter and 45 cm in height (Morris, 1984). The MWM protocol was adapted from Gao *et al.* (Gao *et al*., 2011). Briefly, the pool is filled with water and rendered opaque, using non-toxic tempera paint to form a contrast with the mouse's body for the automated tracking software to detect the mouse. The water is kept at a temperature of 21 ± 1 °C. A transparent plastic square platform (9 cm × 9 cm) is placed approximately 1 cm below the water surface and 10 cm from the edge of the pool. Distal visual cues consist of several wall posters of approximately 0.50 × 0.75 m in size that surround the pool. The whole experiment consists of three phases. (1) Water adaptation trial: to test for stamina and to habituate the mice to the water, (2) Acquisition learning test and probe test, (3) Visual cue tests. Performance in the MWM test was monitored with EthoVision video-tracking system (Noldus Information Technology, Wageningen, The Netherlands).

3.2.6 Statistics

Real-time RT-PCR data and sterol measurements were analyzed by unpaired Student t-test, except in paper IV, where a Mann–Whitney test was used. All the data are shown as means ± SEM. The level of statistical significance was set at $p < 0.05$ for all parameters.

Morris water–maze tests were analyzed with repeated measurements analysis of variance (ANOVA), followed by the Bonferroni post hoc test, using SPSS software. All data are presented as group mean values ±SEM.

4 RESULTS AND DISCUSSION

4.1 ON THE REGULATORY ROLE OF SIDE-CHAIN HYDROXYLATED OXYSTEROLS IN THE BRAIN. LESSONS FROM CYP27A1 TRANSGENIC AND CYP27A1-/- MICE (PAPER I)

4.1.1 Effects of overexpression and knockout of the gene CYP27A1 on cholesterol synthesis

Plasma and brain levels of 27-OH were about 6- and 11- fold higher in transgenic mice than in controls, respectively. 27-OH is a suppressor of cholesterol synthesis under *in vitro* conditions. Therefore, we would expect a suppression of cholesterol synthesis in the brain of transgenic mice. We observed, however, an increase in cholesterol synthesis in the brain of the *CYP27A1tg* mice, as shown by the elevated mRNA levels of *Hmgcr*, *Hmgcs*, and *Srebp2* in the brains of these animals (Figure 4c). In accordance with this, most of the cholesterol precursors in the brain of these mice were also increased (Figure 4a).

There was an approximate 25% reduction in the major cerebral oxysterol 24S-OH. It has been demonstrated previously that 24S-OH is metabolized by CYP27A1 (Bjorkhem *et al.*, 2001), which could be the cause of the low levels of 24S-OH. It is most likely that this reduction in the levels of 24S-OH, which is known to be a suppressor of cholesterol synthesis in neuronal cells (Wang *et al.*, 2008), is the reason for the increased cholesterol synthesis. In spite of the high levels of 27-OH in the brain of transgenic mice compared to the controls, the levels are still much lower than the concentration of 24S-OH in the brain.

There is another possibility that the increased cholesterol synthesis in the brains of the transgenic mice is a compensatory mechanism due to the increased consumption of the brain cholesterol by the overexpressed enzyme. It seems less likely that such a metabolic pathway could be of major importance, since the normal expression of CYP27A1 in the brain is low, and it is believed that most cerebral 27-OH originates from the circulation (Heverin *et al.*, 2005). There was no tendency to reduced levels of cholesterol in *CYP27A1*tg mice (Figure 4a).

Knockout of the *Cyp27a1* gene also resulted in increased cholesterol synthesis in the cortex of brains of *Cyp27a1*$^{-/-}$ mice, as judged by the detection of increased cholesterol precursors (Figure 4b). This result is consistent with previous studies of the brain of this model (Bavner *et al.*, 2010) and has also been confirmed by other groups (Pikuleva I, *unpublished experiments*). The most likely explanation for the increased cholesterol synthesis in these mice is the lack of normal flux of 27-OH into the brain that has a slight inhibitory effect on cholesterol synthesis. It should be pointed out that these mice were treated with cholic acid to compensate for their reduced bile acid synthesis. In theory, bile acid alone could have effects on cholesterol homeostasis in the brain. In the previous work, however, the same results were obtained with both un-treated mice and with mice treated with cholic acid.

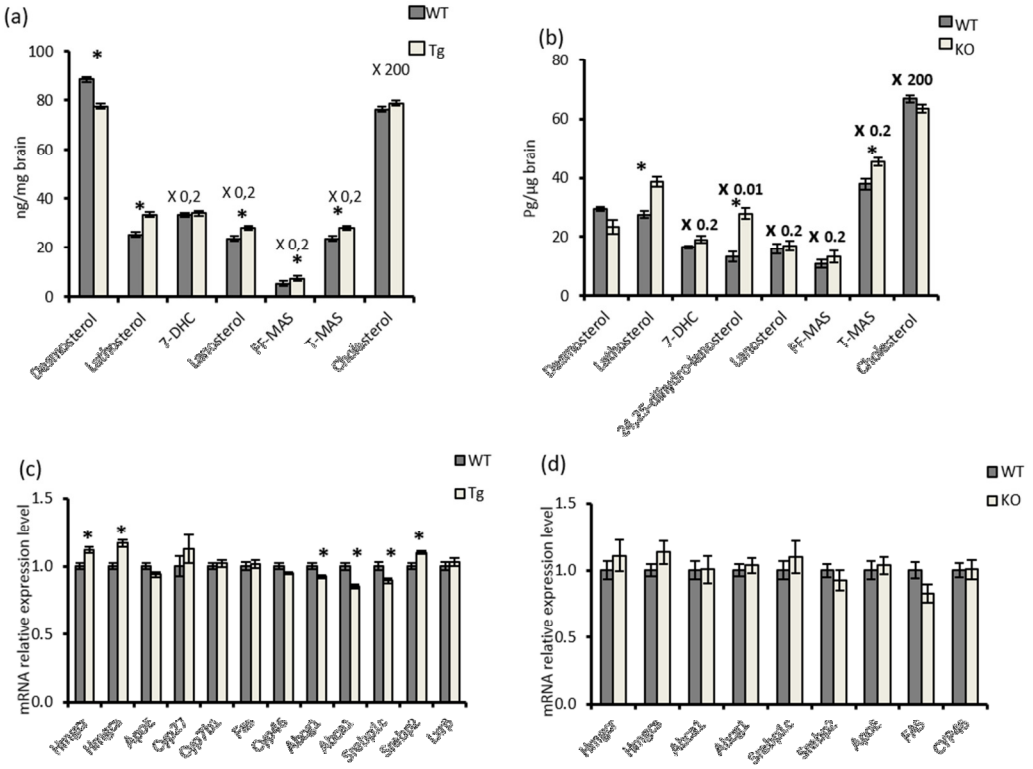

Figure 4. The effects of 27-OH on cholesterol synthesis and LXR-target genes in the adult brain of male mice from different mouse models. *(a), the effect of overexpression of human CYP27A1 on the levels of cholesterol and cholesterol precursors in the brain of CYP27A1 transgenic mice; (b) levels of cholesterol and cholesterol precursors in the brain of Cyp27a1 knockout mice; (c) and (d) mRNA levels of SREBP-regulated and LXR-regulated genes in the brain of CYP27A1 transgenic mice and Cyp27a1 knockout mice, respectively. n = 7-10 in each experiment. Data are presented as mean ± SEM. * P < 0, 05.*

4.1.2 Theoretical model for regulation of cholesterol homeostasis in the brain

The main players in the regulation of cholesterol homeostasis in the brain seem to be HMGCR and CYP46A1. Similar to the situation in the liver, *Hmgcr* is subject to tight regulation in the brain in order to keep the cerebral cholesterol pool at a constant level. On the other hand, *Cyp46a1* is subject to very little regulation at the transcriptional level (Ohyama *et al.*, 2006). Combining the present results with previous results (Lund *et al.*, 2003; Wang *et al.*, 2008; Bavner *et al.*, 2010; Shafaati *et al.*, 2011), we arrived at the regulatory model shown in Figure 5. This model is consistent with all present results, *in vivo* and *in vitro*, but further studies may be needed for confirmation.

Given the fact that 24S-OH is a substrate for the enzyme CYP27A1, we believe that the increased activity of this enzyme is the explanation for the reduced levels of 24S-OH seen in the brain and circulation of our mouse model. It should be emphasized that reduced levels of 24S-OH in the brain may be the consequence not only of increased metabolism in the brain but also outside the brain (Figure 5). Reduced levels of 24S-

OH in the circulation lead to an increased concentration gradient of the oxysterol between the brain and the circulation, which may lead to increased flux from the brain.

After publication of the above results, additional support has been obtained for the contention that the level of 24S-OH in the brain is of importance for cholesterol synthesis in this organ. In a recent study, our group characterized cholesterol homeostasis in the brain of a mouse model with pericyte-deficiency resulting in a leaking blood-brain barrier (Saeed *et al.*, 2014). The defective BBB resulted in a flux of cholesterol from the circulation into the brain and an increased flux of 24S-OH from the brain into the circulation. The latter flux leads to a reduction in the level of 24S-OH in the brain that was reminiscent of the reduced levels of 24S-OH in the brain of our *CYP27A1*tg mice. Also, in this case the reduced levels of 24S-OH were associated with increased cholesterol synthesis in the brain. If the increased levels of 27-OH in the brain of *CYP27A1*tg mice had been the reason for the increased cholesterol synthesis, the same effect would have been expected in the brain of *Cyp7b1*$^{-/-}$ mice. However, neither cholesterol synthesis genes nor cholesterol precursors were affected by the high levels of 27-OH in this mouse model, and the levels of 24S-OH were similar to those of controls (Paper IV, *unpublished results*).

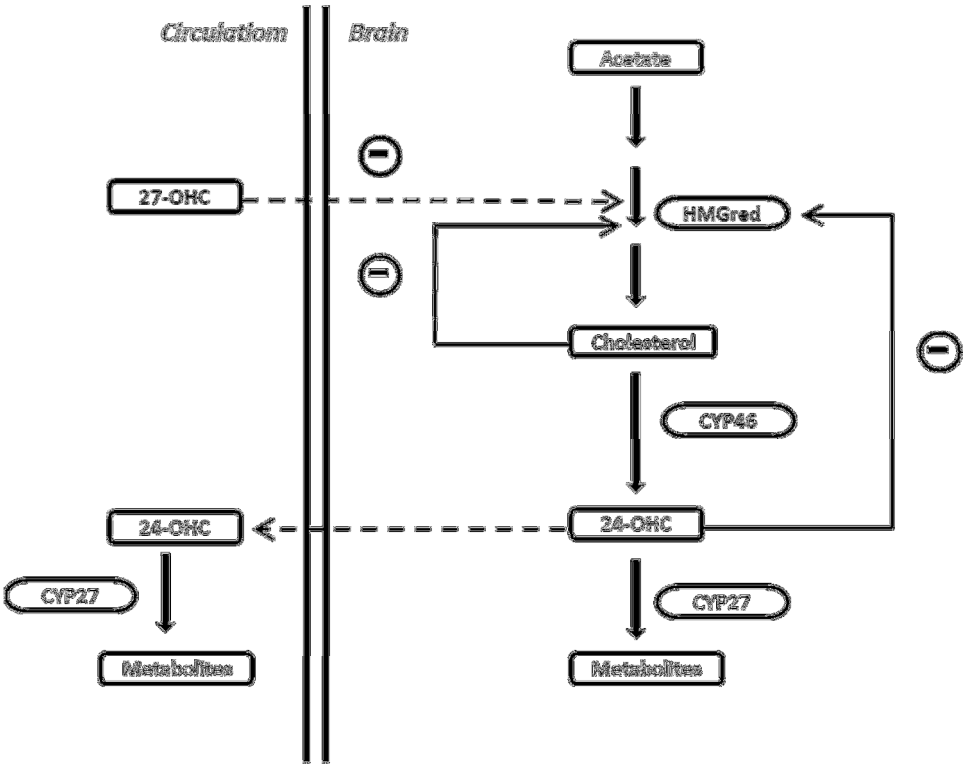

Figure 5. Theoretical model for the regulation of cholesterol homeostasis in the brain.

It should be emphasized that the above model represents a self-regulated system. A primary increase in cholesterol synthesis in the brain leads to some increase in the level

of 24S-OH which counteracts the increased cholesterol synthesis. A primary decrease in production of 24S-OH or an increased leakage of this oxysterol will lead to increased cholesterol synthesis that will counteract the decreased level of 24S-OH. This is a finely tuned system designed to keep the cholesterol levels in the brain as constant as possible. Upregulation or downregulation of the genes involved in the mechanism did not lead to changes in the cholesterol levels measured in the whole brain. It has been reported, however, that overexpression of CYP46 in mouse brain leads to slightly reduced levels of cholesterol in isolated neuronal membranes (Moutinho *et al.*, 2015).

4.1.3 Effects of overexpression and knockout of the gene Cyp27a1 on LXR-targeted genes

In contrast to our expectations, the overexpression of *CYP27A1* led to moderate changes only in the expression of LXR-targeted genes. Some of the LXR-target genes (*Abcg1, Abca1, and Srebpc1*) were slightly decreased by the overexpression rather than increased (Figure 4c). On the other hand, there were no changes in LXR-target genes in the brain of the *Cyp27a1*$^{-/-}$ mice (Figure 4d).

It has been reported that sulphation of 25-OH is an antagonist mechanism for the oxysterol-dependent activation of LXR (Xu *et al.*, 2010). Therefore, we considered the possibility that the opposite effect on LXR target genes that we observed could be due to a sulphated form of 27-OH. In a previous study from our laboratory, we found that the fraction of the total level of the sulphated 27OH in the *CYP27A1*tg mice is similar to that in wild-type mice (Acimovic *et al.*, 2013). Thus, it seems less likely that 27-OH sulphate is of importance for the effects observed.

An alternative explanation is that reduction in the pool of 24S-OH is the cause of the above opposite effect, since 24S-OH is an efficient activator of LXR *in vitro*. In a previous study on mice with high levels of 24S-OH in the brain, there was normal expression of LXR-target genes in the brain (Shafaati *et al.*, 2011). The same result was obtained from another recent study on the pericyte-deficient mouse model that has reduced levels of 24S-OH in the brain (Saeed *et al.*, 2014). The results from these two studies do not support the hypothesis that reduced levels of 24S-OH is the cause of the downregulation of LXR-target genes observed in the brain of the transgenic mice. It should be emphasized that here, we studied the effect in the whole brain and this might mask any specific effects that could have occurred in specific locations of the brain.

In conclusion, our data in this study does not support the contention that 27-OH and 24S-OH are important activators of LXR in the brain, at least under normal conditions. It seems likely, however, that 24S- and 27-OH have a suppressive effect on cholesterol synthesis in the brain.

4.2 ON THE REGULATORY IMPORTANCE OF 27-HYDROXYCHOLESTEROL IN MOUSE LIVER (PAPER II)

4.2.1 Effects of 27-OH on cholesterol synthesis and LXR-target genes in the liver

It has been shown that 27-OH is a strong inhibitor of cholesterol synthesis under *in vitro* conditions (Axelson and Larsson, 1995; Schroepfer, 2000); however, the potential of 27-OH to affect cholesterol synthesis *in vivo*, under normal condition, is still uncertain. In this study, we were interested in investigate the effect of this oxysterol on cholesterol homeostasis and LXR-target genes in the liver of our two mouse models, *Cyp7b1*$^{-/-}$ and *CYP27A1*tg mice, which exhibit high levels of 27-OH in the circulation and tissues; we additionally included a model with undetectable levels of 27-OH in the circulation, *Cyp27a1*$^{-/-}$ mice. The advantage of using two different models with high levels of 27-OH is that, any effect due to 27-OH itself should be seen in both models.

Since 25-OH is also metabolized by CYP7B1, it accumulates in the liver of *Cyp7b1*$^{-/-}$ mice to an extent similar to 27-OH in the liver of *CYP27A1*tg mice. Like the situation with 27-OH, 25-OH is also an efficient suppressor of cholesterol synthesis. Because of this, we expected reduced cholesterol synthesis in the livers of both mouse models with high levels of 27-OH, with the highest inhibition in the liver of the *Cyp7b1*$^{-/-}$ mice. Opposite to our expectations, there was no effect on cholesterol synthesis either in the liver of *Cyp7b1*$^{-/-}$ mice, or in *CYP27A1*tg mice. There were normal mRNA levels of cholesterol-synthesis genes (*Hmgcr, Hmgcs, and Srebp2*) as well as normal levels of most cholesterol precursors in the liver (Figure 6). If 27-OH is an important inhibitor of cholesterol synthesis *in vivo*, then an increase in cholesterol synthesis could be expected in the liver of *Cyp27a1*$^{-/-}$ mice, which is not the case here. Our results are in agreement with previous results (Li-Hawkins *et al.*, 2000), which failed to detect effects on cholesterol synthesis in the liver of *Cyp7b1*$^{-/-}$ mice. The results are also consistent with previous work in our laboratory, which could not demonstrate critical roles for 24S-, 25 or 27-hydroxycholesterol in cholesterol-induced downregulation of *Hmgcr* in mouse liver (Lund *et al.*, 1992). However, the situation might be different under some other pathological conditions. Kannenberg *et al.* reported a marked elevation of 24-, 25-, and 27-OH in fibroblasts from Tanger's disease patients combined with decreased cholesterol synthesis (Kannenberg *et al.*, 2013).

In fact, 27-OH is a less potent activator of the LXR mechanism than other oxysterols. At the same time, it is present at higher concentrations than any other oxysterol in the circulation and all tissues, except for the brain. Therefore, we expected a possible upregulation of LXR-targeted genes in the liver of *Cyp7b1*$^{-/-}$ and *CYP27A1*tg mice, due to the high levels of 27-OH in their circulation and liver. On the contrary, there were no effects in the liver of *Cyp7b1*$^{-/-}$ mice (Figure 6c), while there was a slight upregulation of the genes *Abca1* and *Cyp7b1* in *CYP27A1*tg mice (Figure 6d). If the effect on *Abca1* is due to the high levels of 27-OH, it should be seen in both models. Activation of LXR would result in inhibition of the gene *Cyp7b1* rather than activation (Uppal *et al.*, 2007). A previous study by our group (Shafaati *et al.*, 2011) on the liver of *CYP46A1*tg mice with high levels of 24S-OH in their circulation, also failed to demonstrate any activation of LXR-target genes in spite of the fact that 24S-OH is a potent activator of

LXR (Janowski *et al.*, 1996). There were no differences in the expression of the LXR-target genes between the *Cyp27a1*$^{-/-}$ mice and their controls.

Most of the oxysterols in the liver and circulation (90%) are present in the esterified form (Dzeletovic *et al.*, 1995; Li-Hawkins *et al.*, 2000), and we confirmed this observation here. The presence of 27-OH mainly in the esterified form, which is believed to be the inactive form of the oxysterols, could be the explanation for the failure of 27-OH to exert any effect on cholesterol synthesis and LXR in the livers of *Cyp7b1-/-* and *CYP27A1*tg mice.

The results from different models suggest that 27-OH is unlikely to be an important regulator of cholesterol synthesis and LXR in the liver under basal conditions. However, the situation might be different under some specific pathological conditions.

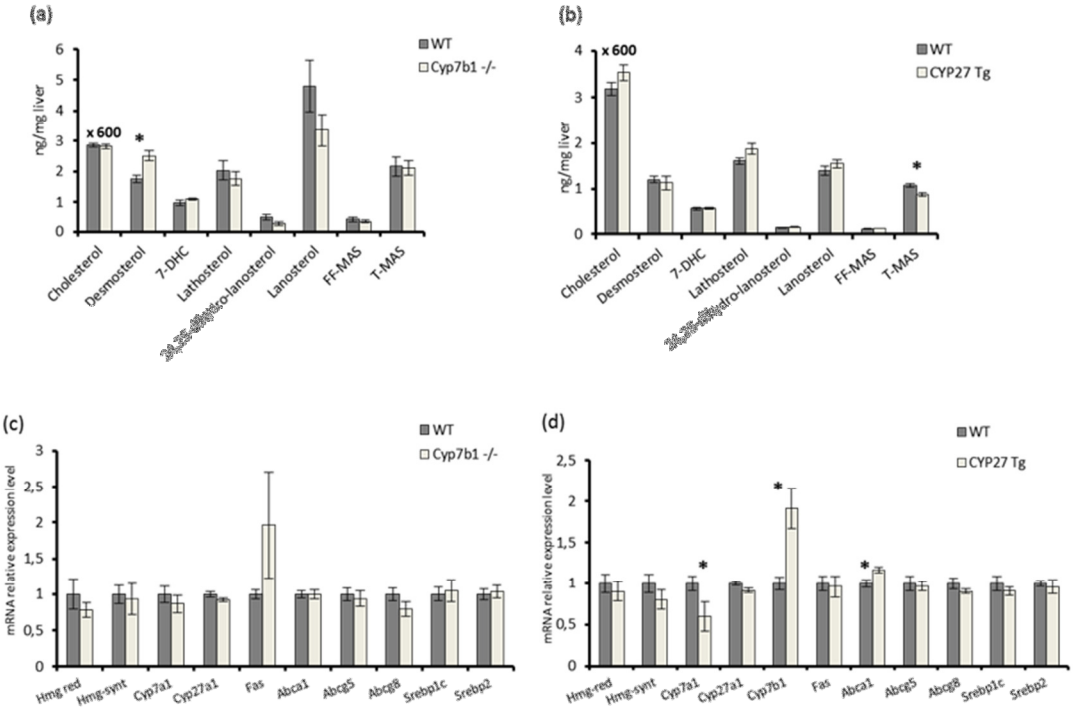

Figure 6. The effect of 27-OH on the cholesterol homeostasis in the liver. *Cholesterol and cholesterol precursors in the liver of Cyp7b1 knockout mice (a), CYP27A1 transgenic mice (b) and mRNA levels of SREBP-regulated and LXR-regulated genes in the liver of Cyp7b1 knockout mice (c), CYP27A1 transgenic mice (d). n = 7-10 in each experiment. Data are presented as mean ± SEM. * P < 0,05.*

4.2.2 Effects of cholesterol feeding on LXR-targeted genes in the liver

It is known that feeding mice with a high dose of dietary cholesterol, which likely elevates oxysterol levels, results in an upregulation of a number of LXR target genes in the liver (Peet *et al.*, 1998; Chen *et al.*, 2007). Chen *et al.* tested the hypothesis that oxysterols are important mediators of the cholesterol-induced activation of the LXR system *in vivo*, using a triple knockout mouse model that lacks the hydroxylases required for the formation of 25-, 24-, and 27-OH (Chen *et al.*, 2007). Since 27-OH is the major oxysterol in the circulation and extra-cerebral tissue, it would be of interest to

evaluate its role in dietary cholesterol-induced activation of LXR. Thus, we repeated the same experiment using a mouse model lacking only the enzyme CYP27A1, maintained on a diet supplemented by cholic acid to compensate for the low cholic acid levels in this model (Repa *et al.*, 2000b).

As expected the expression of the LXR target genes *Abcg5, Abcg8, Srebp1c, Cyp7a1,* and *Lpl*, were significantly increased after 1% cholesterol feeding in wild-type (Figure 7). In the case of *Abcg8, Srebp1c,* and *Lpl*, this increase did not occur in cholic acid-supplemented *Cyp27a1*$^{-/-}$ mice, indicating that 27OH is a mediator of the response to high cholesterol on these three genes. Attempts were made to see if the above changes in mRNA levels of these genes were reflected at the protein levels, but we failed to demonstrate any significant stimulatory effect of cholesterol feeding on the protein levels of ABCG8, or on the activity of LPL enzyme, both in the wild-type mice and in *Cyp27a1*$^{-/-}$ mice.

In the experiment of Chen *et al.*, the stimulatory effect of cholesterol on the expression of the *Srebp1c* gene was not impaired in mutated mice, as was also observed in our experiment (Chen *et al.*, 2007). Furthermore, they observed that the cholesterol-induced effect on *Abcg5* was impaired in their mutated mice. In our study, we observed effects similar to those of Chen *et al.* at the protein level, with a significant increase in Abcg5 protein due to cholesterol treatment in wild-type mice but not in *Cyp27a1*$^{-/-}$ mice. We saw a reduction in the expression of *Fas* as a result of dietary cholesterol treatment in wild-type mice, while no difference was observed in the expression of *Fas* in cholic acid-substituted *Cyp27a1*$^{-/-}$ mice. It is known that *Fas* is an LXR-target gene and is stimulated both by synthetic LXR agonists and by dietary cholesterol (Repa *et al.*, 2000a). Conversely, other studies have shown that treatment with dietary cholesterol reduces *Fas* expression in rodents (Kim *et al.*, 2002; Marseille-Tremblay *et al.*, 2007; Wang *et al.*, 2010). The normal expression of *Fas* in the *Cyp27a1*$^{-/-}$ group is consistent with the possibility that 27-OH is involved in a suppressive effect of cholesterol treatment on the expression of *Fas*.

The results from our work on *Cyp27a1*$^{-/-}$ mice, in combination with the previous work on the triple knockout mice, suggest that 27-OH is a mediator of cholesterol-induced effects on LXR target genes when the mice are challenged with a high load of dietary cholesterol. With respect to the physiological importance of our findings, it should be emphasized that the cholesterol-enriched diet needed to obtain effects on the LXR target genes is highly un-physiological.

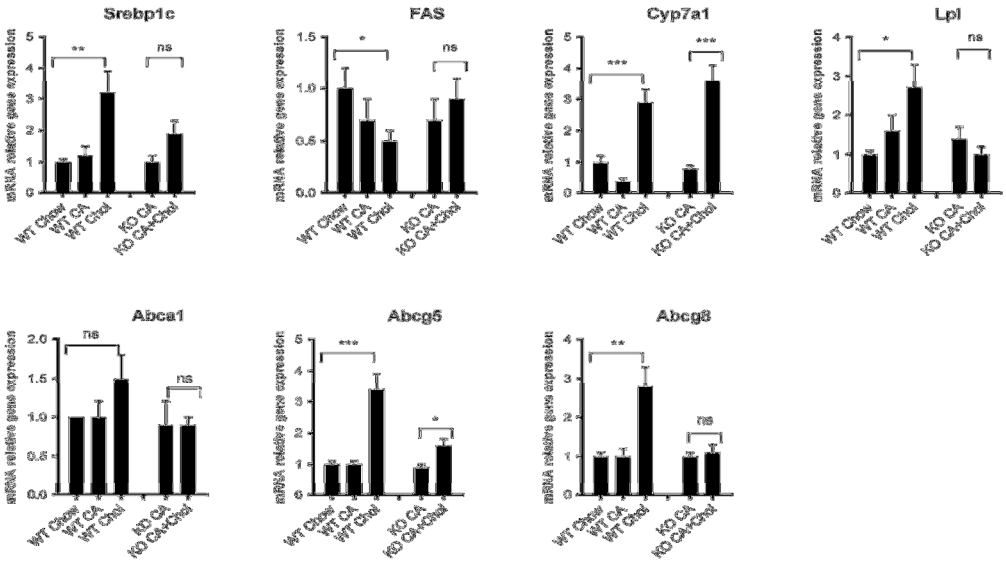

Figure 7. The effect of cholesterol feeding on LXR-target genes in the livers of cyp27a1$^{-/-}$, and wild-type control mice. *Data are presented as mean ± SEM. * P < 0,05.*

4.3 TWO DIFFERENT MOUSE MODELS WITH HIGH LEVELS OF 27-HYDROXYCHOLESTEROL IN KIDNEY AND BRAIN: A COMPARISON (PAPER III)

In this study our aim was to compare the regulatory role of 27-OH on cholesterol homeostasis in the brain and the kidney of two mouse models, *CYP27* A1 overexpressor (*CYP27A1tg*) and *Cyp7b1*$^{-/-}$ mice, which both exhibit markedly elevated 27-OH in their circulation and tissues.

Cyp7b1$^{-/-}$ mice have high levels of 27-OH and 25-OH, while *CYP27A1tg* mice also have high 27-OH but reduced levels of 24S-OH in the brain. We suggested that the increase in cholesterol synthesis detected in the brain of *CYP27A1tg* mice is most likely due to the release of the inhibition on cholesterol synthesis as a consequence of the reduction in levels of 24S-OH. In order to confirm this, we utilized the *Cyp7b1*$^{-/-}$ mouse model. The levels of the side-chain oxysterol 27-OH were similar in the brains of the two strains; however 25-OH, which is also an inhibitor of cholesterol synthesis (Radhakrishnan *et al.*, 2007), is about 14-fold higher in the brains of *Cyp7b1*$^{-/-}$ mice compared to those of *CYP27A1tg* mice. Thus, we expected that the higher amount of the total oxysterols in the brain of the knockout model might be able to induce an inhibitory effect on cholesterol synthesis. Knockout of the gene *Cyp7b1, however*, had no effect on the levels of cholesterol precursors, or on the expression of SREBP-target genes. As described above, the levels of 24S-OH were unchanged in the brains of the *Cyp7b1*$^{-/-}$ mice. In combination with the results of Paper I, these results emphasize the regulatory role of 24S-OH levels on cholesterol synthesis in the brain. It should be emphasized that these levels are markedly higher than those of 27-OH and 25-OH in the brain.

However, the situation is different in the kidney of *Cyp7b1*$^{-/-}$ mice, in which an inhibition of cholesterol synthesis was demonstrated previously (Li-Hawkins *et al.*, 2000). Here, we confirmed this finding as proved by downregulation of the mRNA levels of the *Hmgcr*, the rate limiting enzyme in the cholesterol synthesis pathway (Figure 7c). Also, we confirmed the previous finding that side-chain oxidized oxysterols mainly exist in the form of free oxysterols in the kidney of the *Cyp7b1*$^{-/-}$ mice. Although the free fraction of oxysterol is also elevated in the kidney of *CYP27A1tg* mice (Figure 8a & 8b), there was no inhibition on cholesterol synthesis, as indicated by normal mRNA expression for the gene *Hmgcr* (Figure 8d). For this reason, it seems unlikely that the inhibition of cholesterol synthesis observed in the kidney of the *Cyp7b1*$^{-/-}$ male mice is due to increased levels of free oxysterols. We cannot completely exclude the possibility, however, that the combined suppressive power of the increased levels of both 25-OH and 27-OH in the kidneys of the *Cyp7b1*$^{-/-}$ is larger than the suppressive power of the increased levels of 27-OH in the *CYP27A1tg* mice.

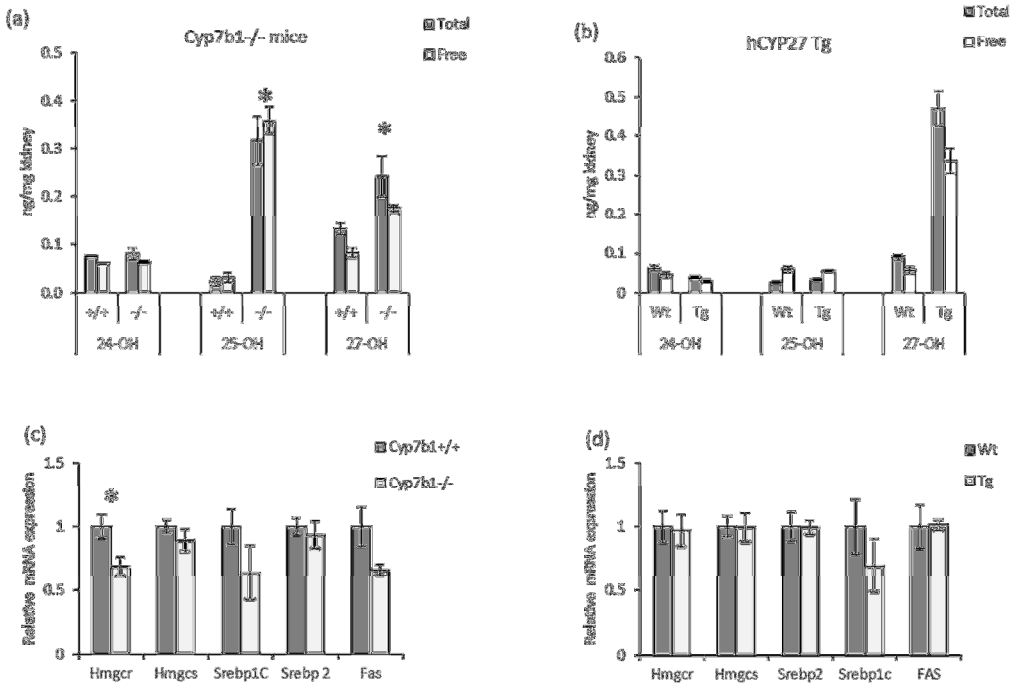

Figure 8. Oxysterols levels and SREBP-target gene expression in the Kidney of Cyp7b1*$^{-/-}$ *and hCYP27a1 overexpressor mice (Tg) and their wild-type controls. *A, free and total 24-, 27-, and 25-hydroxysterols in Cyp7b1*$^{-/-}$*mice. B, free and total 24-, 27-, and 25-hydroxysterols in hCYP27 overexpressor (hCYP27a1 Tg) mice. C, relative mRNA expression of the SREBP-target genes in Cyp7b1*$^{-/-}$ *mice. D, relative mRNA expression of the SREBP-target genes in hCYP27 overexpressor (hCYP27 Tg). n = 3- 4 males in each group. Data are presented as mean ± SEM. * P < 0.05.*

In addition to the metabolism of the oxygenated derivatives of cholesterol, the enzyme CYP7B1 also metabolizes some intermediates in the steroid hormone synthesis pathway such as dehydroepiandrosterone (DHEA), pregnenolone and the estrogen agonist 5α-androstane-3β,17β-diol (3β-Adiol) (Weihua *et al.*, 2002). Combining this

with the fact that *CYP7B1* has a sexual dimorphic pattern of expression (Schwarz *et al.*, 1997; Rose *et al.*, 2001) and is highly expressed in the male kidney, this suggests an important role for this enzyme in the male kidney in a manner which is not clear yet. Therefore, knockout of the gene *Cyp7b1* may result in several consequences such as accumulation of the sex hormone precursor DHEA in the circulation and tissues that might lead to an unbalanced sex hormone synthesis and a disturbance in the estrogen signaling. It has been reported that DHEA inhibits HMGCR expression and activity in a pre-neoplastic liver nodule (Pascale *et al.*, 1995)). Also, it has been reported that disturbances in estrogen signaling may affect cholesterol homeostasis (Choi and Song, 2009)). Further studies are needed to clarify the link between loss of *Cyp7b1*$^{-/-}$ and reduced synthesis of cholesterol in the kidney.

To summarize, our failure to detect an effect on cholesterol synthesis in the brain of the *Cyp7b1*$^{-/-}$ in spite of elevated oxysterols, indicates that 27-OH is not an important regulator of cholesterol homeostasis in the brain of the *Cyp7b1*$^{-/-}$ mice. In combination with the results presented in Paper I, this finding gives further support for a regulatory role of 24S-OH in the brain. On the other hand, the reduced cholesterol synthesis in the kidney of the *Cyp7b1*$^{-/-}$ mice is not likely to be due to the high levels of 27-OH.

4.4 27-HYDROXYCHOLESTEROL MEDIATES NEGATIVE EFFECTS OF DIETARY CHOLESTEROL ON COGNITION IN MICE (PAPER IV)

There is a growing body of data in the literature connecting mid-life hypercholesterolemia to the development of AD later in life (Kivipelto *et al.*, 2001; Whitmer *et al.*, 2005; Solomon *et al.*, 2009; Altman and Rutledge, 2010). It has been shown that dietary cholesterol may result in reduced memory function in mice (Thirumangalakudi *et al.*, 2008). The facts that the serum cholesterol pool is completely isolated from the brain cholesterol pool, and the inability of the serum cholesterol to cross the BBB (Bjorkhem and Meaney, 2004), led us to hypothesis that the cholesterol-induced effect on cognition is mediated by 27-OH, which can pass the BBB. It is known that there is a close relation between levels of cholesterol and 27-OH in the circulation (Babiker *et al.*, 2005). Thus, higher levels of cholesterol in the circulation can be expected to lead to a higher flux of 27-OH into the brain.

To test our hypothesis that the negative effects of dietary cholesterol on cognition are mediated by 27-OH, we used our *Cyp27a1*$^{-/-}$ mouse model and their wild-type controls. Since *Cyp27a1*$^{-/-}$ mice have a reduced production of bile acids and reduced capacity to absorb cholesterol and other lipids (Repa *et al.*, 2000b), their diet was supplemented with 0.05% cholic acid.

Cholesterol feeding to *Cyp27a1*$^{-/-}$ mice resulted in a slower swimming speed compared to the other three groups. Mutations in the *CYP27A1* gene in humans, in contrast with mice, results in a disease called Cerebrotendonous Xanthomatosis (CTX). This disease

is characterized by cholestanol-containing xanthomas in brain and tendon. It has been reported that peripheral neuropathy, in particular of the subtype axonal sensory-motor neuropathy, is common in CTX patients (Chen *et al.*, 2011). We speculated that the combined effect of the lack of the enzyme Cyp27a1and high cholesterol feeding of knockout mice might lead to some peripheral neuropathy with disturbances in motor function. However, this has not been studied before in the *Cyp27a1*$^{-/-}$ mice and further work is needed to test this hypothesis.

During the acquisition part of the Morris water maze test, all groups of mice were able to learn the task as indicated by the decrease in the distance moved over the days (Figure 9a). A significant difference was detected between wild-type mice on cholesterol (wt-chol) and their wild-type controls on chow (wt-chow), since the wt-chol mice needed to move a longer distance to reach the hidden platform. This high cholesterol-induced memory impairment in wild-type mice is in line with the previous studies reporting diminished memory performance in mice fed a high cholesterol diet (Plath *et al.*, 2006; Thirumangalakudi *et al.*, 2008; Ghodke *et al.*, 2012). On the other hand, *Cyp27a1*$^{-/-}$ mice on either cholic acid (KO-CA) or cholesterol plus cholic acid (KO-chol + CA) showed similar distances over the days, indicating that the spatial learning in *Cyp27a1*$^{-/-}$ mice was not affected by the diet (Figure 8a.

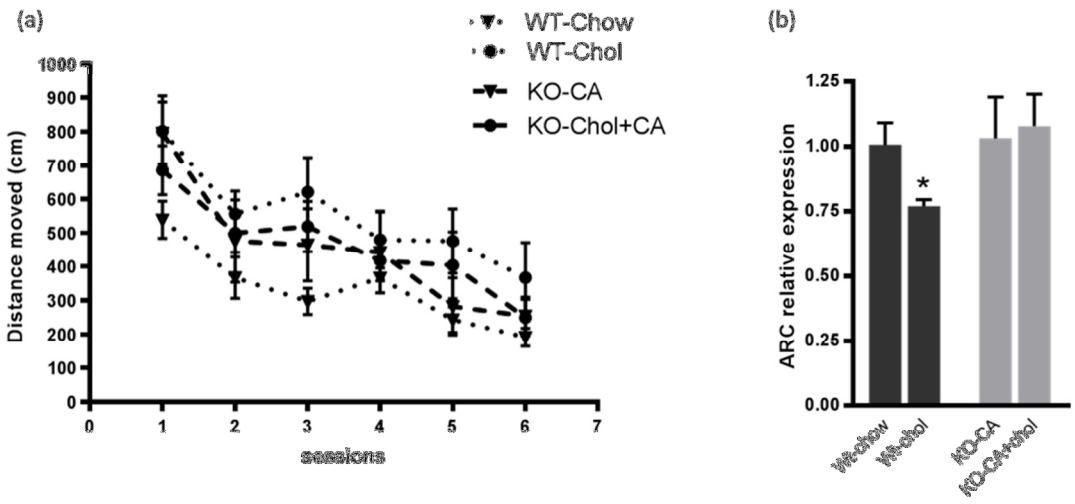

Figure 9. (a) Acquisition phase in MWM test of wild-type and Cyp27KO mice fed on different diets: *data are shown as distance moved (cm) to the hidden platform over 6 training sessions. Results are expressed as mean ± SEM, n = 8–12 animals per group.* ***(b) ARC expression levels in the hippocampus of Cyp27KO and wild-type mice fed on different diets:*** *data are presented as mean ± SEM (*p < 0.05).*

Next, we investigated the effect of high cholesterol feeding on the expression of the "memory protein" activity-regulated cytoskeleton-associated protein, (ARC). This protein is mainly expressed in the hippocampus and is important for the maintenance of long-term potentiation (LTP) and for memory consolidation (Lyford *et al.*, 1995; Guzowski *et al.*, 2000; Plath *et al.*, 2006). Mateos *et al.* reported that high cholesterol feeding decreases ARC protein levels in the mouse brain, and also that addition of 27-

OH decreases the level of ARC in the rat primary hippocampal neurons (Mateos *et al.*, 2009). In accordance with these findings, we detected a downregulation of *Arc* mRNA levels in the hippocampus of wild-type mice treated with cholesterol, while its expression in the hippocampus of the *Cyp27a1*$^{-/-}$ mice was unchanged after cholesterol feeding (Figure 9b). These results are consistent with the possibility that at least part of the negative effects of dietary cholesterol are mediated by ARC through 27-OH. Other mechanisms cannot, however, be excluded. Hypercholesterolemia can induce several inflammatory markers that affect cerebral microcirculation that could lead to cognitive dysfunction (Thirumangalakudi *et al.*, 2008). Interestingly it was recently reported that feeding rabbits a high cholesterol diet is associated with both higher levels of 27-OH in the brain and also hippocampal degeneration in association with altered expression of estrogen receptors (Brooks *et al.*, 2017).

5 CONCLUSIONS

In the present thesis, we investigated the regulatory role of side-chain oxidized oxysterols on cholesterol homeostasis and on cognition using different mouse models.

Paper I – In the brain, both 24S-OH and 27-OH have a suppressive effect on cholesterol synthesis *in vivo*, and 27-OH is not a general activator of LXR.

Paper II – It seems that 27-OH is not an important regulator of cholesterol homeostasis, or an activator of LXR-target genes under basal conditions in the liver. However, when the system is challenged with a high load of dietary cholesterol, the cholesterol-induced effects on some of the LXR target genes in the liver may be mediated by 27-OH.

Paper II – Here, after comparing the effects of high 27-OH on cholesterol synthesis in the brain and kidney of two different models, we confirmed the regulatory importance of 24-OH on cholesterol synthesis in the brain. Factors other than high levels of 27-OH are likely to be important for the reduced cholesterol synthes reported in the kidney of *Cyp7b1*$^{-/-}$ mice.

Paper IV– We show that the negative effects of dietary cholesterol on cognition are mediated by 27-hydroxylation

6 ACKNOWLEDGEMENTS

I would like to thank **Allah** (God) for everything in my life and for His uncountable blessings. I admit that without **Allah** I would not have the necessary strength to succeed and achieve in my life.

I wish to express my warm and sincere thanks and appreciation to everyone who has supported and encouraged me during this journey. I especially wish to express my sincere thanks to:

Ingemar Björkhem, my main supervisor, for accepting me in his research group, introducing me to the world of lipid research, and for always being available and guiding me along this difficult and challenging path of research. I admire his enthusiasm, acumen, and capacity to generate novel scientific ideas and hypotheses. It has been a real pleasure and honour to be one of his students.

Maura Heverin, my co-supervisor, for always being available, supportive, and helpful. Thank you so much for being a great teacher, an amazing guide in the field of research and in working with mice, and for never-ending tips and guidance in work and life.

Angel Cedazo Minguez, my co-supervisor, for a fruitful collaboration, and the warm warm working environment I experienced in his lab.

Amir Babiker, my external mentor, for his friendship and kindness since our first days in Sweden. I feel greatly indebted to him, and without his recommendation I would not have reached this level. I will never forget it.

Maria Olin, **Anita Lövgren Sandblom**, and **Inger Moberg,** for all the effort and help they put in this work. I could not have performed this work without their immense skillful technical assistance, it was highly appreciated. My sincere thanks also go to **Lilian Larsson, Jenny Bernström**, and **Ulla Andersson** for all the help, support, and nice talks.

All my previous and current office-mates, in no specific order: **Dilruba Ahmed, Ahmed Saeed, Osman Ahmed, Treska Hassan, Mirko Minniti, Madeleine Pettersson Bergstrand,** and **Dalma Cricri** for the interesting discussions, continuous help, and for the pleasant moments together.

I also want to thank all of the scientists and group leaders in the Division of Clinical Chemistry: **Paolo Parini, Ulf Diczfalusy**, **Gösta Eggersten**, **Stefan Alexon**, **Anders Helander**, **Jenny Flygare, Matteo Pedrelli**, **Lise-Lotte Vedin**, **Alireza Salehi**, **Tomas Jakobsson**, **Hanna Nylén**, **Camilla Pramfalk,** and **Veronika Tillander.**

Thanks to all of the staff at **PKL4** and **PKL5** for taking care of the mice.

A lot of thanks to the collaborators in **Angel's lab** at the Department of Neurobiology, Care Sciences and Society, with special gratitude to **Silvia Maioli** and **Laura Mateos-Montejo,** for being nice colleagues and for teaching me new laboratory techniques. My thanks are also extended to **Muhammad Al Mustafa Ismail** and **Walid Tajeddinn**, for their supportive attitude.

Neil Portwood, for providing me with invaluable comments during the revision of this thesis.

To every member of the Sudanese community in Stockholm, for the delightful social gatherings and continuous encouragement with special thanks and deep gratitude to all of my friends and their families in Flemingsberg: **Amira, Majda, Dina, Fatima, Wargaa, Lubna**, **Salwa**, **Hiba, Rihab, Samia,** and **Hanaa** for your great support and hospitality.

My dearest friends, in no special order: **Enass Alminawi, Huda Almagli, Amani Abdalla, Nada Omar, Amal Abu Sabaa, Samah Azhari, Randa Abdelhadi, Thowaiba Ibrahim, Randa Alkinain, Abeer Ahmed** for their friendship and all the wonderful times. My best friends in Sudan: **Sarah, Diya**, and **Sally,** for all the nice moments and helping me to get through the difficult times. I would like to thank **Kamal Yassin, Husam Babikir, Yazeed Almalik, Amir Saeed, Salah Shannan, Maisara Alimam, Ashraf Abdelrazig,** and **Siddig Salah,** for their continuous help. My special thanks also go to **Husam Talballa** and **Amal;** I wish you all the best.

I would like to express my great appreciation to all my in-laws for their constant support, faithful prayers, and tender love with special thanks to my mother in-law **Enaam** for keeping me in your prayers all the time.

Tremendous amount of thanks to my entire extended family, especially my parents **Ihsan** and **Mirghani**, my grandmother **YUMMA**, and my lovely aunts: **Suad, Thoraya, Ibtisam Awatif,** and **Shadia**, thank you for your endless love, support, and encouragement, no words in the world would be enough to express my love and gratitude to all of you. My amazing brothers and sisters: **Yousra, Dina, Hiba, Hassan, Reem, Hinda, Abdelrazig, Lina, Mohammed Bakri, Amin, Ahmed Almujtaba, Mohammed Mirghani, and Ola**, thank you for always being there supporting and cheering me every step along the way. Thank you all from the bottom of my heart and I wish you an utmost success wherever you are.

My deepest thanks go to my immediate family: **Mohammed**, for being the best husband and my best friend ever, for your love, enormous support, and encouragement since I have met you. My darling children **Ismail** and **Ahmed**, you are my shining sun that never sets, I love you both so much and wish you a bright future.

This work was supported by grants from the Swedish Science Council, Brain Power, the Swedish Alzheimer's Foundation, Stiftelsen för Gamla Tjänarinnor, and Hjärnfonden. In addition, a partial support was granted by the Ahfad University for Women, and I take this chance to extend enormous amounts of gratitude to **Gasim Badri,** the university president, for his unlimited generosity, and to **Abubekr Abdelazim,** for his kind care.

Zeina Ali

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